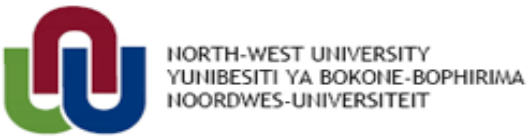


# Elucidation of the Biochemical and Functional Properties of an Unknown Novel Protein from *Arabidopsis thaliana*

**by**

**SELAELO KATLEGO SEHLABANE**

**(17001285)**

**Submitted in fulfilment of the requirements for the degree of a Master of Science in Biology in the Department of Biological Sciences, Faculty of Agriculture, Science and Technology, North-West University, Mafikeng Campus, South Africa**

**Supervisor: Prof. O Ruzvidzo**

**Submission Date: June 2015**

# DECLARATION

I, Selaelo Katlego Sehlabane, solemnly declare that this work entitled '**Elucidation of the Biochemical and Functional Properties of an Unknown Novel Protein from *Arabidopsis thaliana***' is my work and has not been submitted to any institution of learning other than this University for examination or other purposes.

SIGNATURES

..... Date: .....

SELAELO K SEHLABANE

(**Student**)

..... Date: .....

PROF. O RUZVIDZO

(**Supervisor**)

# Table of Contents

# DEDICATION

This work is dedicated to my Family: Lucas Sehlabane, Grace Sehlabane, Mašoto Sehlabane Mampeyane Malatja, Tshegofatso Malatja and my best friend Moirapula Abotseng. Thank you for being my pillars of strength and allowing me to pursue my academics. I honor and appreciate everything you have done for me throughout my studies.

**TO GOD BE THE GLORY**

# ACKNOWLEDGEMENTS

I would like to acknowledge the following individuals and organizations for their contributions towards the completion of this work: My Plant Biotechnology Research Group teammates (2014-2015), thank you to each and every one of you for your assistance and encouragement in the Lab. My supervisor; Prof O. Ruzvidzo, for his patience and encouragement, and for his excellent supervision and strategic mentorship throughout the period of this study. I also would like to thank the technical staff of the Department of Biology for their support and facilitation. The National Research Foundation (NRF) for financially supporting this study.

# DEFINITION OF TERMS

**Adenylate Cyclases (ACs):** Enzymes capable of converting adenine 5′-triphosphate (ATP) to cyclic 3′,5′-adenosine monophosphate (cAMP).

**Enzyme Immunoassay:** An antibody based diagnostic technique used in molecular biology for the qualitative and quantitative detection of specific biological molecules.

**Guanylate Cyclases (GCs):** Enzymes capable of converting guanine 5′-triphosphate (ATP) to cyclic 3′,5′-guanosine monophosphate (cAMP).

**Mass Spectrometry:** A biochemical method used to detect biological molecules according to their quantities and molecular weights.

**Primers:** Short synthetic nucleic acid sequences capable of forming base pairs with a complementary template RNA/DNA strand and facilitating its specific amplification.

**Proteomes:** A collection of cellular proteins whose expression levels are co-regulated by a single and specific signaling molecule.

**Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR):** A molecular method used to amplify a short RNA segment into a DNA product termed copy DNA (cDNA) using an RNA-dependent DNA polymerase enzyme.

**Second Messenger:** A biological molecule capable of transmitting external cellular signals within the cell for the development of appropriate cellular responses through regulated gene expressional and metabolic events.

**Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE):** A technique used in molecular biology to separate different protein molecules according to their sizes and migration levels in a polyacrylamide gel system subjected to a strong electrical field.

# LIST OF ABBREVIATIONS

**AC:** Adenylate cyclase

**ANOVA:** A one-way analysis of variance

**ATP:** Adenosine 5′-triphosphate

**BLAST:** Basic Local Alignment Searching Tool

**cAMP:** Cyclic 3′,5′-adenosine monophosphate

**cGMP:** Cyclic 3′,5′-guanosine monophosphate

**IPTG:** Isopropyl-β-D-thiogalactopyranoside

**LB:** Luria-Bertani

**Ni-NTA:** Nickel-nitrilotriacetic acid

**NO:** Nitric oxide

**OD:** Optical density

**Rpm:** Revolutions per minute

**RT-PCR:** Reverse transcriptase polymerase chain reaction

**SDS-PAGE:** Sodium dodecyl sulphate polyacrylamide gel electrophoresis

**ST:** Sodium Tris-HCl

**STAND:** Signal transduction ATPases with numerous domains

**TAIR:** The Arabidopsis Information Resource

**UV:** Ultraviolet

**YT:** Yeast-tryptone

# LIST OF FIGURES

# LIST OF TABLES

# ABSTRACT

Adenylate cyclases (ACs) are a special group of enzymes capable of catalyzing the conversion of adenosine 5′-triphosphate (ATP) into the signaling molecule cyclic 3′,5′-adenosine monophosphate (cAMP), which in turn acts as a second messenger in various cellular and metabolic pathways. Apparently, while the presence of ACs and their functional roles in animals and prokaryotes have since been well-documented, their presence and/or functional roles in higher plants has somewhat remained a matter of serious debates and controversy. Notably and in a recent BLAST search of the Arabidopsis genome using a 14-mer motif with specificity for ATP binding and catalysis, an AC-like protein coded for by the At3g21465 gene has been identified. However, even though the AC-like protein does contain the AC catalytic core motif, it notably has not yet been shown to possess any known putative AC catalytic function and/or share any similarities with any annotated and/or experimentally confirmed ACs, but instead, it only appears to be transcriptionally up-regulated in response to biotic stress factors. Therefore in an attempt to test and determine whether this putative protein candidate has any functional AC activity, total mRNA of the 4-6 weeks old *Arabidopsis thaliana* plants was extracted and used as a template for the complementary synthesis and amplification of a 384 bp AC-like gene fragment via a specialized Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) system. The amplified fragment was then cloned into a pTrcHis2-TOPO expression vector and the resultant recombinant expression vector eventually transformed into chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS expression host cells. Positive clones were determined by confirmatory PCR and further validated by nucleotide-specific sequencing. The 18.0 kDa C-terminus His-tagged recombinant AC-like protein was then over-expressed following an induction with isopropyl-β-D-1-thiogalactopyranoside (1 mM, IPTG) and purified over a nickel-nitrilotriacetic acid (Ni-NTA) affinity matrix system. The endogenous and *in vitro* AC activities of the resultant recombinant AC-like protein were then tested via a cAMP-linked enzyme immunoassaying system while its inherent *in vivo* AC activity was also concurrently tested via a complementation testing system using the *cya*A SP850 mutant *Escherichia coli* cells. Results from these three independent assays collectively indicated that the AC-like protein encoded for by the At3g21465 gene from *A. thaliana* possesses the endogenous, *in vitro* and *in vivo* AC activities, and thus unequivocally confirming it as a *bona fide* higher plant AC molecule with a possible cAMP-mediated signaling system.

# CHAPTER ONE

## INTRODUCTION AND LITERATURE REVIEW

### 1.1 Introduction

Food security and the global climate change are two interrelated factors that have since begun to raise numerous and growing concerns throughout the world. The effects of global climate change have to this day seriously impacted onto food security even though the whole aspect of crop production does not necessarily depend on the changing climatic conditions such as temperature increases, droughts and floods but also on the agricultural sector's ability to re-assess its practices and activities to optimize the efficiency of food production and adapt through changes in technology, coupled with the proper management of water availability, soil quality, and crop selection (McCalla, 1999). Apparently, biotechnology can be used as one of the most promising tools in solving problems associated with the agricultural production as it has the potential to effectively address specific problems such as increasing crop productivity, improving crop diversification, enhancing food nutritional value as well as reducing the various environmental stress factors on agricultural production.

In this study, we specifically focused onto a recently identified putative *Arabidopsis thaliana* adenylate cyclase-like (ACL) gene (At3g21465) annotated at the NCBI http://www.ncbi.nlm.nih.gov/protein/51968402 site. This gene does contain an adenylate cyclase (AC) core motif, but has no any other annotated domains and/or been shown to possess any known putative AC catalytic function and more so, does not share any similarity with any annotated and/or experimentally confirmed ACs, but instead, only appears to be transcriptionally up-regulated in response to biotic stress factors (Gehring, 2010). Apparently, this gene fragment is notably known to be located in the mitochondrion and is differentially expressed in guard cells - cells that are responsible for transpiration in plant leaves and therefore, playing a very important physiological role in the proper regulation and/or maintenance of water and gas levels in plants during extreme weather conditions such as floods and droughts (TAIR:http://www.arabidopsis.org).

Generally and in most studied organisms including plants, the structural system of ACs is typically composed of three distinct protein components namely; a catalytic subunit which converts the substrate ATP to cAMP, a guanine nucleotide-binding protein which mediates hormonal activation, and a hormone receptor (Rodbell, 1980). The physiological regulation of the activity of this enzyme and hence of the intracellular levels of cAMP, occurs primarily through interactions initiated by hormones binding to receptors and mediated by the guanine nucleotide-binding protein.

Surprisingly, not much is known in higher plants about ACs or their product cAMP as is in animals and lower eukaryotes although currently, there are already four experimentally tested and functionally confirmed ACs in plants. These are the *Zea mays* pollen signaling protein responsible for the polarized pollen tube growth (Moutinho *et al*., 2001), the *Arabidopsis thaliana* pentatricopeptide repeat protein responsible for pathogen responses and gene expressions (Ruzvidzo *et al*., 2013), the *Nicotiana benthamiana* adenylyl cyclase protein responsible for the tabtoxinine-β-lactam-induced cell deaths during wildfire diseases (Ito *et al*., 2014), and the *Hippeastrum hybridum* adenylyl cyclase protein involved in stress signaling (Swiezawska *et al*., 2014).

Thus besides identifying an additional AC in higher plants, our study on the At3g21465 gene was also aimed at providing us with some possible understanding of how exactly do plants respond to the various biotic and abiotic stress factors and therefore, further facilitating us in our current knowledge of possibly improving both the aspects of crop production and national food security.

## 1.2 Literature Review

### 1.2.1 Cyclic Adenosine Monophosphate (cAMP) and Adenylate Cyclases (ACs)

A substantial amount of research has so far been done on the 3′,5′-cyclic adenosine monophosphate (cAMP), which controls the metabolism, cell growth and differentiation of both prokaryotic and eukaryotic organisms. Naturally, cAMP mediates cell responses to various environmental signals, and thereby acting as an intracellular second messenger. The discovery

of the biological importance of this molecule mainly resulted from studies on the action of adrenaline and glucagon in liver tissues (Rall *et al*., 1957).

Arguably, cAMP is one of the most extensively studied second messengers in all organisms, and as has already been mentioned earlier on, this molecule is produced from ATP by the enzyme AC. Investigations onto the nature of its distribution have unequivocally shown that it is not just confined to mammalian tissues but also to fungal, bacterial and animal tissues (Perlman and Pastan, 1969). An example of a fungal species where cAMP signaling has been found to be essential for the growth and differentiation of pseudohyphae (chains of elongated cells) is the *Saccharomyces cerevisiae* (brewer's or baker's yeast) (Kronstad *et al.*, 1998). In this fungus, the activity of ACs is regulated primarily by its *Ras* homologues, the *Ras1p* and *Ras2p* (DeFeo-Jones *et al.*, 1985; Field *et al.*, 1988).

More investigations were also conducted on the Mycobacteria genus, many of which are pathogenic to humans, and the species *Mycobacterium tuberculosis*, which is the sole causative agent of tuberculosis (TB). A large number of common human deaths were then shown to be as a result of TB infection and its nexus with the human immunodeficiency virus (HIV), thus further resulting in a relatively high number of fatalities in the acquired immune deficiency syndrome (AIDS) patients (Smith, 2003). In these investigations, it was also found that relatively high levels of intra and extracellular cAMP were produced during the mycobacterial growth *in vitro*, which then pointed out towards the high cAMP-synthesizing capacity of the adenylate cyclases that are naturally encoded in the genomes of Mycobacteria (Lowrie *et al*., 1975). Conceivably, the cAMP levels in Mycobacteria were found to be approximately 100-200-fold higher than those reported for *Escherichia coli* (*E. coli*) grown in glycerol (a growth medium in which the cAMP levels of *E. coli* are essentially highest) (Padh and Venkitasubramanian, 1976).

In the mammalian system, cAMP is implicated as a mediator of the actions of many hormones and its role as a mediator of hormone action has been extensively reviewed by Robison *et al*. in 1968. These studies however, do not give a detailed characterization of the ACs activities and their regulations, or provide major evidence on their involvement in the control of important responses of higher plant cells to environmental stimuli. Therefore and as a result of this, it is

not surprising that many research groups have this far, put considerable efforts into finding ACs particularly in *Arabidopsis thaliana,* the first higher plant and the third ever multicellular organism whose genome has been integrally sequenced in the year 2000, which sequentially then positively contributed towards the build-up of a reference data base for the field of plant genomics (The *Arabidopsis* Genome Initiative, 2000).

Virtually, information on ACs and cAMP has not been readily available in plants as has been in animals and lower eukaryotes, due to the fact that the levels of cAMP detected in plants were significantly low (<20 pmol/g fresh weight) (Ashton and Polya, 1978) as compared to those found in animals (>250 pmol/g wet weight) (Butcher *et al*., 1968) and therefore, firm conclusions could not be reached due to the lack of conducive results in plant assays, probably due to some methodological problems encountered in previous years, when methods developed for the analysis of cAMP in mammalian tissues were simply adopted for plants.

However, subsequent studies done on the *Medicago sativa* L. (alfalfa) showed that the adenylate cyclase activity could be partially characterized in roots. The results indicated the existence in alfalfa roots of an adenylate cyclase activity with two important properties; firstly, a non-sedimentable enzyme activity and secondly, that it could be activated by carbon dioxide ions and calmodulin. Furthermore, research done on *Vicia faba* reported that cAMP could promote stomatal opening and could also remove the effects of external $Ca^{2+}$ and abscisic acid (ABA) by the cytochemical staining methods (Curvetto and Delmastro, 1990). This study further showed that the accumulation of $K^{+}$ ions in stomatal guard cells was also associated with the cAMP-induced increases of stomatal apertures (Curvetto and Delmastro, 1994).

Forskolin, a cardioactive diterpene (Lindner *et al*., 1978) was isolated from the roots of *Coleus forskohlii* (Bhat *et al*., 1977) and has been described as an activator of adenylate cyclases in membranes and intact cells (Seamon *et al*., 1981***)***. The activation by forskolin is rapid and reversible and does not require guanine nucleotides (Metzger and Lindner, 1981). Additionally, research was also done in *Lilium longiflorum* and in this study, it was found that the elongation of pollen tubes in pistils after self-pollination exhibited a strong gametophytic self-incompatibility which essentially, was promoted by cAMP and also equally promoted by activators (forskolin and choleratoxin) of adenylate cyclases and inhibitors (3-isobutyl-1-

methylxanthine and pertussis) of cyclic nucleotide phosphodiesterases (Tezuka *et al*., 1993). Furthermore, cAMP enhanced choline acetyltransferase (ChAT) activity and suppressed acetylcholinesterase (AChE) activity in the pistils, suggesting that the results were closely correlated with self-incompatibility in the *Lilium longiflorum* (Tsuruha *et al*., 2001).

## 1.3 Problem Statement

Nowadays, the urgent need to use rational approaches to develop crop plants with increased stress responses and tolerance has led to an impressive body of work in the areas of plant genetics, plant physiology, plant biochemistry and plant molecular biology, and a realization that only an integrated and systems-based approach could possibly deliver effective biotechnological solutions (Stuhmer *et al*., 1989). Hence, since proteins that systemically affect homeostasis in plants are a target candidate group for biotechnology, one such molecule termed an adenylate cyclase-like protein from *Arabidopsis thaliana* (AC-like) was hereby extensively studied in this project. A complete functional characterization of this annotated Arabidopsis protein was strongly sought to positively contribute towards a better understanding of the general mechanisms by which plants respond and adapt to stressful environmental conditions. This understanding in turn, would then further advance our current scientific knowledge on plant genes responsible for environmental stress responses and adaptation mechanisms. All in all, the whole understanding would also then broaden our overall knowledge of the trends through which environmental stresses do affect plants.

## 1.4 Aim of the Research Study

The major aim and research question of this study was to gain insight into ACs and establish if there was any other additional functional AC in higher plants besides the only and already experimentally confirmed; *Zea mays* pollen signaling protein (Moutinho *et al*., 2001), *Arabidopsis thaliana* pentatricopeptide repeat protein (Ruzvidzo *et al*., 2013), *Nicotiana benthamiana* adenylyl cyclase protein (Ito *et al*., 2014), and *Hippeastrum hybridum* adenylyl cyclase protein (Swiezawska *et al*., 2014) and if so, whether such an AC was capable of mediating plant responses to different environmental signals particularly the biotic and abiotic stress factors. This study then specifically addressed the sought research question by focusing

onto an investigation of the possible functional properties of a recently identified and bioinformatically annotated AC-like gene (At3g21465) in the Arabidopsis genome (Gehring, 2010).

## 1.5 Objectives of the Research

The following specific objectives were set to address the sought aim and main question of this research study:

1. To isolate the annotated AC-like (At3g21465) gene from *Arabidopsis thaliana.*
2. To clone the AC-like gene fragment into a stable and viable heterologous prokaryotic expression system.
3. To optimize strategies for the expression and purification regimes of the recombinant AC-like protein.
4. To determine the adenylate cyclase enzymatic activity of the recombinant AC-like protein.
5. To further characterize the enzymatic activities of this AC-like recombinant protein.

## 1.6 Significance of the Research Study

Upon a successful completion of this sought research study, the following significances were set to be accomplished:

1. The identification and establishment of yet another additional functional higher plant AC besides the only and currently known *Zea mays* pollen signaling protein (Moutinho *et al*., 2001), *Arabidopsis thaliana* pentatricopeptide repeat protein (Ruzvidzo *et al*., 2013), *Nicotiana benthamiana* adenylyl cyclase protein (Ito *et al*., 2014), and *Hippeastrum hybridum* adenylyl cyclase protein (Swiezawska *et al*., 2014).
2. A relatively better understanding of the general mechanisms by which plants respond and adapt to stressful environmental conditions.
3. A potential contribution towards the moderate integrated management of both biotic and abiotic stressful conditions of agronomically important crops in South Africa.

# CHAPTER TWO

## RESEARCH METHODOLOGIES

## 2.1 Cloning of the AC-like Gene Fragment

### 2.1.1 Plant Re-generation and Growth Conditions

*Arabidopsis thaliana* ecotype Columbia seeds were germinated on Murashige and Skoog (MS) plate media. Briefly, about 100 seeds were successively washed three times with 70% (v/v) ethanol in a 1.5 Eppendorf tube. The washing alcohol was discarded and the seeds were then further similarly washed with 500 µl of the seed sterilization buffer (0.1% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) commercial bleach). Thereafter, the seeds were then successively rinsed (5 times) with 1 ml of sterile distilled water before being suspended in 500 µl of the sterile distilled water. The seeds were then stratified (for uniform germination) at 4ºC for 3 days. The stratified seeds were then germinated in MS petri dishes and allowed to grow for two weeks in the growth chamber, under greenhouse conditions adjusted to 23/16 day/night for periods of 8/16 hours night/day at 10 000 light lux. The germinated seedlings were then transplanted into potting soil (50% (w/w) peat based soil and 50% (w/w) vermiculite), watered with sterile distilled water, covered with plastic cling wrap to retain moisture, and then kept in the growth chamber to grow for a further 2-4 weeks. The annotated AC-like gene was eventually isolated from these 4-6 week old Arabidopsis plants.

### 2.1.2 Designing and Acquisition of Sequence-specific Primers

The nucleotide sequences of the At3g21465 gene encoding the putative AC-like protein (Figure 2.1) was retrieved from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) web site. Two sequence-specific primers were then manually designed based on this At3g21465 gene sequence and specifically, to flank its annotated AC catalytic motif (Figure 2.1). The designed primer sequences were then sent to the Inqaba Biotechnological Sciences (Pretoria, RSA) for chemical synthesis and subsequent supply.

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | A | K | R | G | D | T | E | S | L | W | N | V | D | K | L | R | S | E | T |
| Y | T | Q | H | T | L | S | G | A | F | S | C | A | K | G | F | L | L | E | H |
| K | P | E | E | A | A | A | V | I | Q | I | I | C | Q | A | Y | P | D | E | K |
| K | S | A | L | E | A | E | F | K | K | L | V | N | E | W | S | V | D | I | I |
| K | H | Q | N | E | Q | D | K | K | D | V | A | A | S | L | K | S | D | I | P |
| A | M | V | N | A | L | V | N | S | G | L | R | V | R | V | D | L | N | E | L |
| N | K | N | E | A | L | L | S | - | | | | | | | | | | | |

**At3g21465AC FWD**
gCT gCC AAA AgA ggA gAC ACA gAg TCg TTA

**At3g21465AC REV**
gCT AAg AAg AgC TTC ATT CTT gTT TAA CTC

**Figure 2.1:** The complete amino acid sequences of the AC-like protein showing its annotated AC catalytic centre (green highlight and underlined) and the priming sites of this targeted catalytic centre (blue highlights and underlined). The two manually designed sequence-specific primers for this targeted AC catalytic centre are also given (yellow highlights).

### 2.1.3 Isolation and Amplification of the AC-like Gene Fragment

The AC-like gene fragment was synthesized from the total mRNA obtained by harvesting about 0.1-0.2 g of plant leaf material from the 4-6 weeks Arabidopsis plants using the Thermo Scientific GeneJET Plant RNA Purification Mini Kit, and according to the manufacturer's protocol (Thermo Scientific Inc., Massachusetts, USA). Briefly, about 100 mg of fresh leaf tissue was flash-frozen in liquid nitrogen followed by its thorough grinding with a pestle and mortar into a fine powder. The tissue powder was immediately transferred into 500 µl of plant lysis solution in a 1.5 ml Eppendorf tube and the tube was vortexed at high speed for 20 seconds. The mixture was then incubated at 56ºC for 3 minutes and later centrifuged at 20,000x*g* in a LSE High Speed Microcentrifuge (Corning Inc., Amsterdam, Netherlands). The supernatant was transferred to a clean 1.5 ml Eppendorf tube and mixed by pipetting with 250 µl of 96% (v/v) ethanol. The mixture was transferred to a purification column in a collection tube and centrifuged for 1 minute at 12,000x*g*. The flow-through was discarded while the column and collection tube were re-assembled. About 700 µl of wash buffer 1 was added to the purification column and the column then centrifuged for 1 minute at 12,000x*g*. The purification column was placed in a new and clean 1.5 ml collection tube and 500 µl of wash buffer 2 was added to the column. The column was centrifuged for 1 minute at 12,000x*g*. After the flow-through was discarded, the wash step was repeated one more time. The column was then transferred to an

RNAse-free 1.5 ml collection tube and the mRNA then eventually eluted by adding 50 µl of nuclease-free water directly onto the column membrane and centrifuging for 1 minute at 12,000xg.

The extracted total mRNA was then used as a template to generate copy DNA (cDNA). Together with the acquired sequence-specific primers, the generated cDNA was subsequently used to amplify the targeted AC-like gene fragment in a reverse transcriptase - polymerase chain reaction (RT-PCR) system using the Thermo Scientific Verso 1-Step RT-PCR Reedy system and as was instructed by the manufacturer's protocol (Thermo Scientific Inc., Massachusetts, USA). The used reaction mixtures and cycling conditions are shown below in Tables 2.1 and 2.2.

**Table 2.1:** Components of an RT-PCR reaction mix in a final reaction volume of 50 µl.

| Composition | Volume (µl) | Final Concentration |
|---|---|---|
| Verso Enzyme Mix | 1 | |
| 1-Step RT-PCR Reddy Master Mix | 25 | 1X |
| Forward Primer (10 µM) | 1 | 200 nM |
| Reverse Primer(10 µM) | 1 | 200 nM |
| RT Enhancer | 2.5 | |
| Water (PCR Grade) | 19.5 | |
| Template (mRNA) | 1 | 1 ng |
| Total Volume | 50 | |

**Table 2.2:** The 1-step RT-PCR thermal cycling program used for amplification of the AC-like fragment gene.

| Step | Temperature (ºC) | Time | Cycles |
|---|---|---|---|
| cDNA Synthesis | 50 | 15 minutes | 1 |
| Thermo-start Activation | 95 | 15 minutes | 1 |
| Denaturing | 95 | 20 seconds | 45 |
| Annealing | 65 | 30 seconds | |
| Extension | 72 | 1 minute | |
| Final Extension | 72 | 5 minutes | 1 |

### 2.1.4 Agarose Gel Electrophoresis of the Amplified AC-like Gene Fragment

The amplified RT-PCR product was resolved on a 1% (w/v) agarose gel supplemented with 0.5 µg/ml ethidium bromide. All samples were resolved against a 100 bp Gene-Ruler DNA ladder and immersed in 1X TBE buffer at 80 volts and a constant current of 250 mA for 50 minutes. The gel was then visualized under UV light using the UV 2000 Trans-illuminator System (Bio-Rad Laboratories Inc., California, USA). The images were then finally captured using a Complete ChemiDoc Imaging System (Bio-Rad Laboratories Inc., California, USA).

### 2.1.5 Cloning of the Amplified AC-like Gene Fragment

#### 2.1.5.1 Addition of 3′-adenines Overhangs

A total volume of 1 µl *Taq* polymerase was added to 40 µl of the RT-PCR product reaction and the mixture then incubated at 72ºC for 10 minutes on a C1000 Thermo-cycler System (Bio-Rad Laboratories Inc., California, USA). The resultant reaction mixture was then kept on ice for further use.

#### 2.1.5.2 Ligation of the Adenylated AC-like Gene Insert into the pTrcHis2-TOPO Vector

An aliquot of 4 µl was collected from the adenylated AC-like gene fragment reaction mixture and then transferred into a fresh PCR tube containing 1 µl of the pTrcHis2-TOPO expression vector (Invitrogen, Carlsbad, USA) and the two solutions were then gently mixed by pipette swirling. The ligation mixture was then incubated at room temperature for 5 minutes before its subsequent use for the transformation process of competent *Escherichia coli* expression cells.

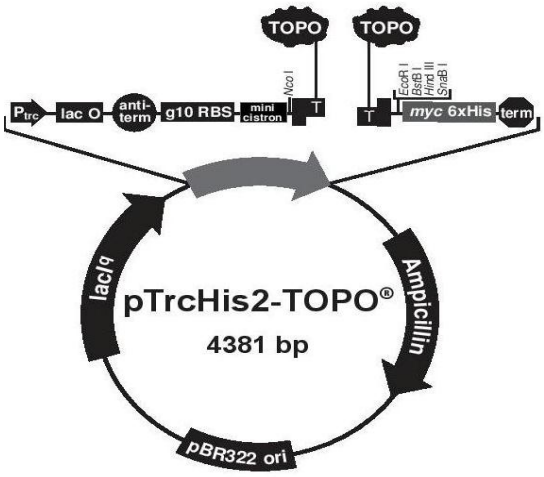


**Figure 2.2: Commercially acquired pTrcHis2-TOPO vector for cloning of AC-like PCR product:** The illustration shows expression and purification features of the plasmid such as the $\mathbf{P_{trc}}$ promoter for high level expression with forward and reverse priming sites for both restriction sites *Nco* I and *Eco* RI within a multiple cloning site. There is also a point of origin to facilitate replication of the plasmid in bacteria cells such as *E. coli*. In addition, there is an ampicillin resistant gene that allows for screening of positive recombinants. For purification purposes, the vector expresses a recombinant 6-Histidine fusion protein that can be affinity purified on positively-charged chromatographic columns. (Adapted from www.lifetechnologies.com).

### 2.1.5.3 Transformation of the Competent One Shot TOPO 10 *E. coli* Cells with the pTrcHis2-TOPO:AC-like Fragment Construct

Immediately after the ligation process, about 2 µl of the ligation mixture (pTrcHis2-TOPO:AC-like fragment construct) was added into an ice-cold Eppendorf tube containing about 40 µl of the chemically competent One Shot TOPO 10 *E. coli* cells. The reaction mixture was gently mixed and incubated on ice for 30 minutes. The reaction mixture was then heat-shocked on a dry-bath heating block at 42ºC for 30 seconds before being immediately incubated on ice for 5 minutes. Subsequently, the reaction mixture was supplemented with 250 µl of SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $Mg_2SO_4$ and 20 mM glucose) and incubated in a shaker at 37ºC at 200 rpm for 30 minutes (this incubation step is always carried out so as to allow cells to produce the β-lactamase enzyme, which will later and during the selection process, detoxify ampicillin). The mixture was then plated (80 µl and 20 µl) onto two Luria Bertani (LB) agar plates (1% (w/v) agar, 1% (w/v)

tryptone powder, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) supplemented with 50 µg/ml ampicillin and 0.5% (w/v) glucose. The plates were then incubated at 37ºC overnight.

#### 2.1.5.4 Extraction of the pTrcHis2-TOPO:AC-like Expression Construct from the Transformed One Shot TOPO 10 *E. coli* Cells

Single colonies from the selective plated LB plates were used to prepare overnight cultures, whereby individual colonies were picked and separately inoculated into 10 ml falcon tubes of the double strength yeast-tryptone (2YT) media (0.8% (w/v) tryptone powder, 0.5% (w/v) yeast extract and 0.25% (w/v) NaCl) supplemented with 50 µg/ml ampicillin and 0.5% (w/v) glucose. The falcon tubes were then incubated overnight at 37ºC shaking at 200 rpm. The next morning, cells were then harvested by centrifuging at 6 800x*g* for 5 minutes at room temperature, and the supernatant discarded. Plasmid extraction was then performed at room temperature using the GeneJET Plasmid Miniprep Kit and according to the manufacturer's instructions (Thermo Fisher Scientific Inc., California, USA).

Briefly, the pelleted cells were resuspended (through pipetting up and down) into 250 µl of a resuspension solution supplemented with RNase until no cell clumps had remained. The resuspended solution was then transferred into a sterile 1.5 Eppendorf tube. This was followed by the addition of 250 µl of lysis solution and mixing thoroughly by inverting the tube 4-6 times or until the solution has become viscous and slightly clear. Thereafter, 350 µl of the neutralization solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times. The cell debris was then pelleted out by centrifugation for 5 minutes at 16 300x*g* while the supernatant was carefully transferred into the supplied GeneJET spin column. The spin column fitted into a collection tube was then centrifuged for 1 minute at 16 300x*g* and its flow-through discarded while the column was re-placed into the same collection tube. About 500 µl of wash solution (diluted with 96% (v/v) ethanol prior to first use) was added to the spin column and the column was further centrifuged for 30-60 seconds. The flow-through was discarded while the column was placed back into the same collection tube. The washing procedure was repeated twice more before the spin column was centrifuged for an extra 1 minute to remove any residual wash solution. The washed and semi-dried GeneJET spin column was then fitted into a new and sterile 1.5 ml Eppendorf tube where the plasmid DNA was then eluted

by the adding 50 µl of the elution buffer to the center of the GeneJET spin column membrane followed by an incubation of 2 minutes at room temperature and centrifugation at 16 300x*g* for 2 minutes. The used column was then discarded while the purified plasmid DNA (pTrcHis2-TOPO:AC-like fragment construct) was stored at -20ºC for further use.

### 2.1.6 Analysis of Positive Clones

Confirmation of positive clones was carried out by conventional PCR and in accordance with the standard MyTaq Mix protocol (Bioline, London, UK) to check if the AC-like gene insert was successfully ligated into the pTrcHis2-TOPO vector and also in the correct orientation. The reaction mixtures for the two processes are shown in Tables 2.3 and 2.4 respectively while the associated thermal cycling conditions for both processes are shown in Table 2.5 below.

**Table 2.3:** Reaction components of a PCR reaction mixture to confirm the successful ligation of the AC-like gene insert into the pTrcHis2-TOPO expression vector.

| **Component** | **Volume (µl)** |
|---|---|
| Template (10 ng DNA) | 1 |
| Insert Primers (20 µM each) | 1 |
| MyTaq Reddy Mix, (2X) | 25 |
| Water (sdH2O) | Up to 50 |

**Table 2.4:** Reaction components of a PCR reaction mixture to confirm the correct orientation of the AC-like gene insert in the pTrcHis2-TOPO expression vector.

| **Component** | **Volume (µl)** |
|---|---|
| Template (10 ng DNA) | 1 |
| Insert Forward and Vector Reverse Primers (20 µM each) | 1 |
| MyTaq Reddy Mix, (2X) | 25 |
| Water (sdH2O) | Up to 50 |

**Table 2.5:** The thermal cycling program for the step by step assessment profile of the successful ligation and correct orientation of the AC-like gene insert into the pTrcHis2-TOPO expression vector.

| Step | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Initial Denaturation | 95 | 1 minute | 1 |
| Denaturing | 95 | 15 seconds | 25-35 |
| Annealing | 65 | 15 seconds | |
| Extension | 72 | 10 seconds | |

### 2.1.6.1 Agarose Gel Electrophoresis of the AC-like Gene Fragment from the Confirmed Clones

The PCR products from both Tables 2.3 and 2.4 were resolved on an ethidium bromide-stained (0.5 µg/ml) 1% (w/v) agarose gel immersed in a 1X TBE buffer at 80 volts, 250 mA for 50 minutes and the samples were then resolved against a 100 bp Gene-Ruler DNA ladder. Visualization was undertaken under UV light using a UV 2000 Trans-illuminator System (Bio-Rad Laboratories Inc., California, USA) and pictures of the resultant images eventually captured by a Complete ChemiDoc Imaging System (Bio-Rad Laboratories Inc., California, USA). Double amplification of the targeted AC-like gene insert in both reaction samples of Tables 2.3 and 2.4 was therefore correspondingly confirming its successful and positive cloning into the pTrcHis2-TOPO expression vector. In addition, all the successfully confirmed clones were also sent over to the Inqaba Biotechnological Sciences (Pretoria, RSA) for validation through nucleotide-specific sequencing.

### 2.1.7 Transformation of the Chemically Competent *E. cloni* EXPRESS BL21 (DE3) pLysS Cells with the AC-like Fusion Expression Construct

After positively confirming the cloning process of the AC-like gene fragment into the pTrcHis2-TOPO expression vector, its resultant expression construct (pTrcHis2-TOPO:AC-like) was then used to transform some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS expression cells. This transformation was carried out in a sterile Eppendorf tube and in accordance with the manufacturer's protocol and instructions (Lucigen, Wisconsin, USA), whereby 1 µl of the pTrcHis2-TOPO:AC-like expression construct was aseptically added to 100 µl of the ice-cold *E.*

*cloni* EXPRESS BL21 (DE3) pLysS cells. The mixture was then incubated on ice for 30 minutes before being heat-shocked for 45 seconds at 42ºC and immediately placed on ice for 5 minutes. The reaction mixture was then supplemented with 960 µl of the expression recovery medium and incubated in a shaker at 37ºC at 200 rpm for 1 hour. The transformation reaction mixture was then plated onto LB agar plates supplemented with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The plates were then incubated overnight at 37ºC.

## 2.2 Expression and Affinity Purification of the Recombinant AC-like Protein

### 2.2.1 Recombinant Expression

A transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cell colony harboring the pTrcHis2-TOPO:AC-like expression construct was inoculated into 10 ml of 2YT media supplemented with 0.5% (w/v) glucose, 34 µg/ml chloramphenicol and 100 µg/ml ampicillin in a 15 ml falcon tube. The falcon tube was incubated overnight at 37ºC, shaking at 200 rpm. On the subsequent day, 200 µl of the overnight culture was then inoculated into fresh 20 ml 2YT media containing 34 µg/ml chloramphenicol, 100 µg/ml ampicillin and 0.5% (w/v) glucose. The culture was incubated at 37ºC, shaking at 200 rpm and until an $OD_{600}$ of 0.6 was reached and as was measured by the Hekios Spectrophotometer (Merck, Gauteng, RSA). Immediately, the culture was split into two tubes each of 10 µl. One culture was induced to express the intended AC-like recombinant protein by adding 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich Corp., Missouri, USA) while the other culture was left un-induced (control). The split cultures were then shaken in an incubator (200 rpm) at 37ºC for 3 hours. After the 3 hours, the cultures were then centrifuged at 8 000x*g* for 5 minutes to pellet out the cells. The pelleted cells were then stored at -20ºC before being analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as well as used for the other further downstream processes.

### 2.2.2 Affinity Purification

Protein purification was performed under native non-denaturation conditions since the recombinant AC-like protein was found to be wholly expressed as a soluble fusion product. The pelleted out bacterial cells carrying the expressed recombinant AC-like protein were re-

suspended in 5 ml phosphate saline (PBS) buffer (140 mM NaCl, 3 mM KCl, 4 mM $Na_2HPO_4.2H_2O$, 1.5 mM $KH_2PO_4$ (pH: 8.0)) supplemented with 10 mM imidazole and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The sample was then sonicated for 6 minutes (10 seconds pulsing and 10 seconds chilling cycles) to solubilize the cell contents. The solubilized cell contents were then centrifuged at 9 200x*g* for 10 minutes and the supernatant was subsequently collected and kept on ice as a cleared lysate. Alongside this, about 500 µl of the nickel-nitrilotriacetic acid (Ni-NTA) resins (Thermo Scientific Inc., Rockford, USA) were washed twice with 2 ml sterile distilled water on a rotary mixer for 5 minutes. The Ni-NTA resins were then equilibrated with 1 ml of PBS supplemented with 10 mM imidazole and 0.5 mM PMSF. The equilibrated resins were then pelleted out through a low speed centrifugation for 15 seconds, and the supernatant was discarded. The generated cleared lysate was then mixed (binding) with the equilibrated Ni-NTA resins on a rotary mixer for 1 hour at 4ºC. After an hour, the resins were washed three times with 5 ml PBS supplemented with 10 mM imidazole and 0.5 mM PMSF, and each wash and the bound resins kept for further and subsequent resolution by SDS-PAGE on a 12% (w/v) gel.

### 2.2.3 Elution of the AC-like Recombinant Protein

The bound and fully purified AC-like recombinant protein was eluted off the Ni-NTA resins through the addition of 2 ml elution buffer (200 mM NaCl, 50 mM Tris-Cl (pH: 8.0), 250 mM imidazole, 0.5 mM PMSF, and 20% (v/v) glycerol) and allowing the mixture to settle for 10 minutes. The resultant supernatant containing the eluted AC-like protein was then collected and stored at 4ºC for further downstream use and analysis by SDS-PAGE.

### 2.2.4 Concentration and Desalting of the Recombinant AC-like Protein

The eluted and purified AC-like recombinant protein was freed from the buffering salts and excess water by pouring the 2 ml eluent into the upper chamber of the Spin-X UF de-salting and concentrating device (Corning Corp., New York, USA). The device was then centrifuged at 2 540x*g* at 4ºC for 4 hours or until the final volume was down to 100 µl. The concentrated and de-salted protein fraction was then removed from the device and transferred to a new Epperndorf tube. Protein concentration was then subsequently determined by a 2000 Nanodrop

Spectrophotometer (Thermo Scientific Inc., Califonia, USA) and the recovered protein sample stored at -20ºC.

## 2.3 Activity Assaying

### 2.3.1 Determination of Endogenous Activity of the Recombinant AC-like Protein

An overnight culture of cells confirmed to be harbouring the recombinant pTrcHis2-TOPO:AC-like expression construct was prepared using 200 µl of their glycerol stock to inoculate 10 ml of fresh 2YT media supplemented with 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. The culture was grown at 37ºC in a shaking incubator at 200 rpm. On the next day, fresh 20 ml of the 2YT media containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were then inoculated with 1 ml of the overnight culture and incubated at 37ºC in a shaker at 200 rpm and up until the $OD_{600}$ had reached 0.6. The culture was immediately placed on ice and split into four portions of 3 ml each.

Protein expression was then induced by the addition of 1 mM IPTG into three cultures and one tube was left un-induced (control). From two of the three induced cultures, one culture was supplemented with 100 µM forskolin (Sigma-Aldrich Corp., Missouri, USA) and the other culture with 100 µM 2′,5′-dideoxyadenosine (Sigma-Aldrich Corp., Missouri, USA). All four cell cultures were then incubated at 37ºC for a further 2 hours before cells were harvested by centrifugation at 9 200x*g* for 10 minutes. The harvested cells were then lysed in 1 ml lysis buffer 1 (Amersham Healthcare Inc., California, USA) supplemented with 2 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich Corp., Missouri, USA) to inhibit phosphodiesterases. In actual sense, the samples were shaken at 100 rpm, at 37ºC for 30 minutes in on orbital shaker to intensify the cell lysis process. The samples were then centrifuged at 9 200x*g* for 5 minutes and their lysates transferred into fresh Eppendorf tubes where 200 µl of the lysis buffer 2 (Amersham Healthcare Inc., California, USA) was added and mixed.

After that, 220 µl of the mixture was then transferred into a fresh Eppendorf where 11 µl of the acetylating reagent (Sigma-Aldrich Corp., Missouri, USA) was also added before the mixture was gently mixed by pulsing. The endogenous cAMP content from each of the generated lysates was then measured using a cAMP-linked enzyme immunoassaying kit (Catalog # CA201)

following its acetylation version of its protocol and in accordance with the manufacturer's manual (Sigma-Aldrich Corp., Missouri, USA). The measurements were taken using a Microplate Reader (Labtech International Limited, East Sussex, UK) at 405 nm and all obtained results were then subjected to the statistical analysis of variance (ANOVA) in triplicate sets.

### 2.3.2 Complementation Testing of the Recombinant AC-like Protein

Some competent mutant *E. coli* host cells, the *cya*A SP850 strain (Coli Genetic Stock Center, Yale University, Connecticut, USA) were divided into two portions. The first portion was transformed with the pTrcHis2-TOPO:AC-like expression construct, while the other portion was left un-transformed (control). Alongside this, a MacConkey agar plate supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG (Sigma-Aldrich Corp., Missouri, USA) was also prepared and then sub-divided into 3 quadrants using a permanent marker. The first quadrant was left un-streaked (no *cya*A cells), the second quadrant was streaked with the non-transformed *cya*A mutant cells while the last quadrant was streaked with the c*ya*A mutant cells transformed with the pTrcHis2-TOPO:AC-like expression construct. The plate was then inverted and incubated at 37ºC for 40 hours. After the incubation, all quadrants were then visually inspected for the various phenotypic characteristics. In this case, a reddish or deep purple color on the transformed mutant cells would indicate a positive AC activity for the cloned and recombinantly expressed AC-like protein.

### 2.3.3 Determination of the *In Vitro* Enzymatic Activity of the Recombinant AC-like Protein

The *in vitro* enzymatic activity of the purified AC-like recombinant protein was determined by assessing its ability to convert ATP to cAMP in a Tris-buffered system. To determine this activity, 10 µg of the recombinant AC-like protein were incubated with 1 mM ATP, 2 mM IBMX, and 5 mM $Mg^{2+}$ in a final volume of 200 µl 50 mM Tris- HCl (pH 8.0). Other additives like GTP, $Ca^{2+}$, $F^{-}$, $HCO_3^{2-}$, and $Mn^{2+}$ were also tested in order to determine their direct or indirect influences onto the catalytic activities of the purified AC-like recombinant. Residual cAMP levels resulting from the non-AC activity were also measured in tubes that contained the incubation medium but no protein added. All reaction incubations were performed for 20 minutes at 25ºC and terminated by the addition of 10 mM of ethylene di-amine tetra-acetic acid

(EDTA). Tubes were then boiled for 3 minutes, cooled on ice for 2 minutes before being centrifuged at 9 200x*g* for 3 minutes. The resulting supernatant was then assayed for cAMP content using the cAMP-linked enzyme immunoassaying kit (Catalog # CA201) following its acetylation version of its protocol and as was specifically described by the manufacturer's manual (Sigma-Aldrich Corp., Missouri, USA). All results and outcomes were then subjected to the statistical analysis of variance (ANOVA) in triplet forms.

# CHAPTER THREE

## RESULTS AND INTERPRETATIONS

### 3.1 Molecular Isolation of the AC-like Gene Fragment

The AC-like gene fragment (At3g21465) was isolated by amplifying its sequences from the total *Arabidopsis thaliana* mRNA via a specialized RT-PCR system (Thermo Scientific Inc., Burlington, Canada) and using the manually designed sequence-specific forward and reverse primers (Fig 2.1). The successfully isolated AC-like gene fragment (Fig 3.1) was then cloned into a pTrcHis2-TOPO expression vector to form a pTrcHis2-TOPO:AC-like expression construct which subsequently, was then used to transform some chemically competent One Shot TOPO 10 *E. coli* BL21 (DE3) pLysS cells.

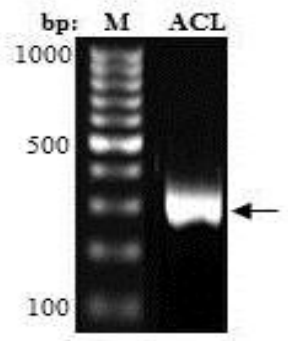


**Figure 3.1: Molecular Isolation of the AC-like Gene Fragment.** The targeted and desired AC-like gene fragment (At3g21465) was isolated by amplifying it from the total *Arabidopsis thaliana* mRNA via a specialized RT-PCR system followed by its resolution on a 1% (w/v) agarose gel. Lane 1 (M) represents the 100 bp Gene-Ruler ladder (Thermo Scientific Inc., Burlington, Canada) while lane 2 (ACL) represents the amplified AC-like gene fragment. The arrow marks the resultant and successfully isolated AC-like gene fragment.

### 3.2 Confirmation of the Cloning Success of the AC-Like Gene Fragment

After the successfully isolated AC-like gene fragment in Figure 3.1 above was ligated into the pTrcHis2-TOPO expression vector, its cloning success was then checked and verified by standard PCR using a specialized MyTaq reaction system. Furthermore, this cloning success was also confirmed through the nucleotide-specific sequencing at the Inqaba Biotechnological Sciences (Pretoria, RSA). The successfully cloned and molecularly confirmed AC-like gene fragment is shown below (Fig 3.2).

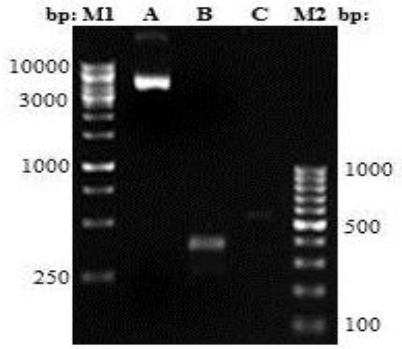


**Figure 3.2: Confirmation of the Cloning Success of the AC-like Gene Fragment.** The AC-like gene fragment ligated into the pTrcHis2-TOPO expression vector was re-amplified with its own sequence-specific primers as well as by one of its own primers and one of the vector primers followed by its resolution on a 1% (w/v) agarose gel system. Lane 1 (M1) represents the 100 bp Gene-Ruler ladder (Thermo Scientific Inc., Burlington, Canada), lane 2 (A) represents the pTrcHis2-TOPO:AC-like gene expression construct, lane 3 (B) represents the AC-like gene fragment amplified with its own sequence-specific primers, lane 4 (C) represents the AC-like gene fragment amplified with its own forward primer and the vector reverse primer, while lane 5 (M2) represents the 1 kb Gene-Ruler ladder (Thermo Scientific Inc., Burlington, Canada).

## 3.3 Partial Expression of the Recombinant AC-like Protein

When the cloning system of the AC-like gene fragment into the pTrcHis2-TOPO expression vector was successfully confirmed (section 3.2 above), its recombinant expression construct (the pTrcHis2-TOPO:AC-like gene fragment) was then used to transform some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells followed by a partial expression of the respective and desired AC-like recombinant (Fig 3.3).

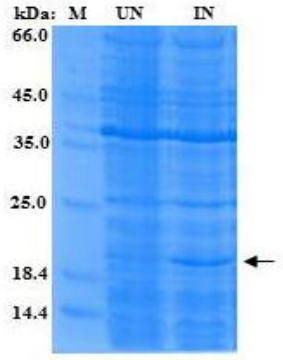


**Figure 3.1.1: Partial Expression of the Recombinant AC-like Protein.** An SDS-PAGE of protein fractions expressed in chemically competent *E. cloni* EXPRESS BL21 (DE3) plysS cells transformed with the pTrcHis2-TOPO:AC-like fusion construct, where lane 1 (M) is the unstained low molecular weight marker (Fermenters Int., Burlington, Canada), while lane 2 (UN**)** represents the un-induced control cell culture and lane 3 (IN) representing the bacterial cell culture treated with 1 mM IPTG to induce the partial expression of the targeted and desired recombinant AC-like protein. The arrow marks the partially expressed recombinant AC-like protein.

Figure 3.4 below presents the exact amino acid sequences of the cloned and partially expressed recombinant AC-like protein. This sequence, together with its associated C-terminus histidine tag (His-tag) amino acid sequences from the pTrcHis2-TOPO expression vector, will produce a resultant fusion recombinant product of approximately 18.0 kDa and as has already been shown in Figure 3.3 above.


**Figure 3.3.2: The Amino Acid Sequences of the Truncated AC-like Protein from *Arabidopsis thaliana*.** The residues marked in red and underlined represent the annotated AC catalytic center in this putative protein while those highlighted in blue and underlined represent the forward and reverse priming sites.

## 3.4 Determination of the Endogenous Adenylate Cyclase Activity of the Recombinant AC-like Protein

After successfully expressing the targeted recombinant AC-like protein in the transformed chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells, its ability to generate cAMP from ATP within these prokaryotic expression systems (endogenously) was assessed and determined and also under various culturing and growth conditions. The resultant endogenous adenylate cyclase activity of this partially expressed recombinant AC-like protein is presented below in Figure 3.5.

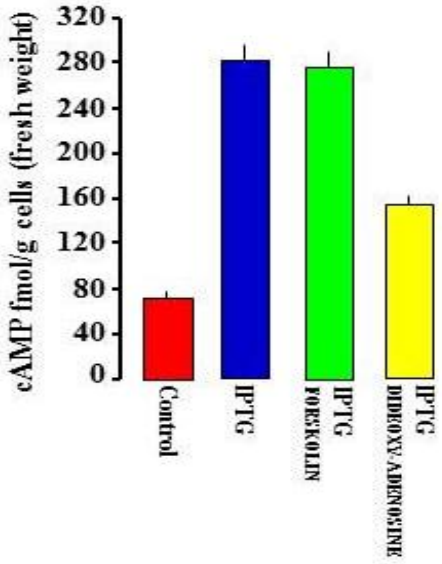


**Figure 3.4: Determination of the Endogenous Adenylate Cyclase Activity of the Recombinant AC-like Protein.** Some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells transformed with the successfully designed pTrcHis2-TOPO:AC-like gene fragment expression construct were grown under various culturing conditions followed by an assessment of the expressed AC-like recombinant protein's ability to generate cAMP from ATP under these different culturing conditions in these prokaryotic systems. For all cultures, the generated cAMP levels were determined using a cAMP-linked enzyme immunoassaying system (Sigma-Aldrich Inc., Missouri, USA) and based on its acetylation protocol. Analyses were undertaken in triplicate sets (n = 3), where error bars represent the standard errors of the replicate means.

## 3.5 Determination of the *In Vivo* Adenylate Cyclase Activity of the Recombinant AC-like Protein

After the expressed recombinant AC-like protein had successfully demonstrated its ability to generate cAMP from ATP within the transformed *E. cloni* EXPRESS BL21 (DE3) pLysS cells, its expression construct (the pTrcHis2-TOPO:AC-like gene fragment) was then used to transform some chemically competent SP850 *cya*A *E. coli* mutant cells that systematically cannot ferment lactose as a result of their inability to generate the most required cAMP. Consequently, the transformed SP850 *cya*A cells were then assessed for their ability to now ferment lactose and as a result of their rescuing by the then inherent and expressed recombinant AC-like protein (Fig 3. 5).

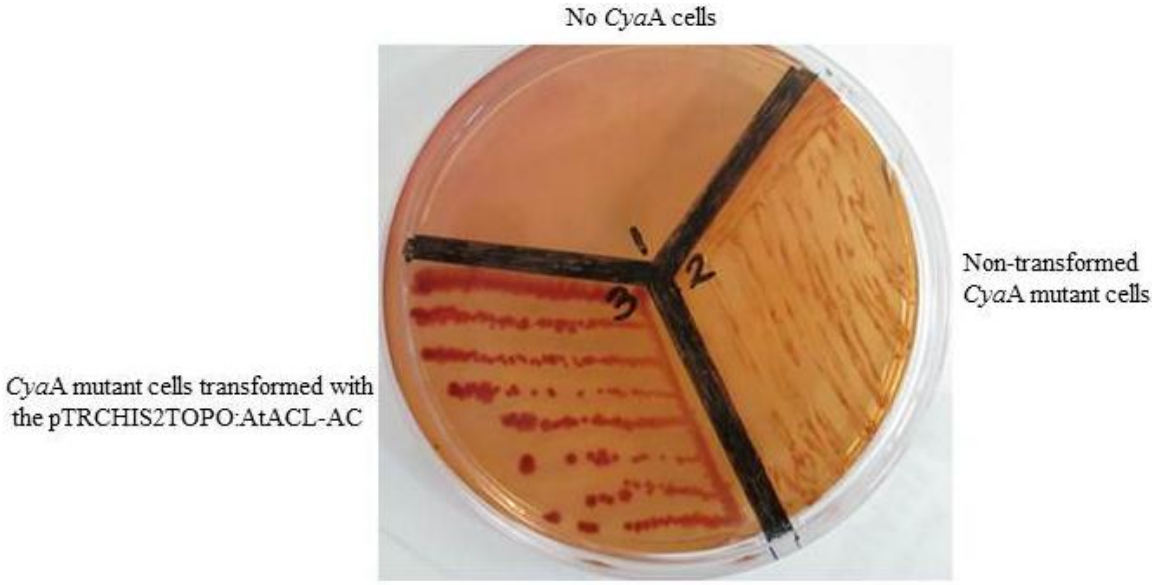


**Figure 3.5: Determination of the *In Vivo* Adenylate Cyclase Activity of the Recombinant AC-like Protein.** Different chemically competent *E. coli cya*A mutant cells were plated onto three different quadrants of a MacConkey agar supplemented with 15 µg/ml kanamycin and 0.1 mM IPTG and incubated for 40 hours at 37ºC. Quadrant 1 of the plate contains no cells, quadrant 2 contains the non-transformed *E. coli cya*A mutant cells, while quadrant 3 contains the *E. coli cya*A mutant cells transformed with the pTrcHis2-TOPO:AC-like expression construct. Cells in quadrant 2 are non-lactose fermenters and therefore produce white or yellowish colonies. Cells in quadrant 3 have now picked a deep red or purplish phenotype – a characteristic signifying their acquired ability to now ferment lactose and as a result of the then presence and functional activity of the now inherent AC-like protein, which now essentially generates the most required cAMP for this process.

## 3.6 Affinity Purification of the Recombinant AC-like Protein

After the cloned and expressed recombinant AC-like protein had unequivocally demonstrated both its endogenous (section 3.4) and *in vivo* (section 3.5) adenylate cyclase activities, it was then purified off the rest of the other bacterial proteins via an Ni-NTA affinity matrix system (Sigma-Aldrich Inc., Missouri, USA) so as to further functionally characterize it. This purification regime (and its resultant partial purification profile) is systematically presented in Figure 3.6 below.

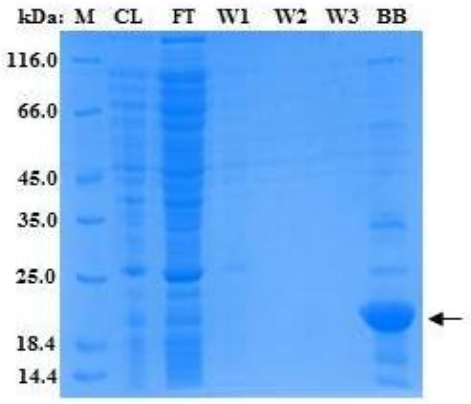


**Figure 3.6: Affinity Purification of the Recombinant AC-like Protein.** An SDS PAGE of the recombinant AC-like protein fractions collected at the different stages of its purification process using the Ni-NTA affinity matrix system (Sigma-Aldrich Inc., Missouri, USA). In this presentation, M represents the unstained low molecular weight marker (Fermenters Int., Burlington, Canada), CL represents the cleared cellular lysate before its passage through the Ni-NTA affinity matrix, (FT) represents the flow-through of the cleared lysate after it was passed through the Ni-NTA matrix, (W1) is the first wash of the bound AC-like recombinant protein onto the Ni-NTA affinity matrix with the wash buffer, (W2 and W3) are the second and third washes respectively, and (BB) is the purified and bound AC-like recombinant protein. The arrow marks the resultant and partially purified AC-like recombinant protein product.

## 3.7 Chemical Elution of the Recombinant AC-like Protein

After the expressed recombinant AC-like protein was partially purified from its associated bacterial protein contaminants (section 3.6), it was then eventually eluted off the Ni-NTA affinity matrix so that its functional enzymatic activities as a pure protein could then be subsequently assessed *in vitro*. The finally eluted and purely purified protein fraction of the AC-like recombinant product is shown below (Figure 3.7).

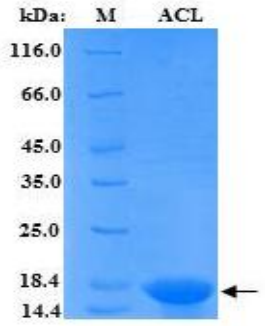


**Figure 3.7: Chemical Elution of the Recombinant AC-like Protein.** An SDS-PAGE of the purified recombinant AC-like protein fraction eluted off the Ni-NTA affinity matrix with 250 mM imidazole, where M is the unstained low molecular weight marker (Fermenters Int., Burlington, Canada) and the arrow is marking the resultant purified and chemically eluted recombinant AC-like protein fraction.

## 3.8 Determination of the *In Vitro* Adenylate Cyclase Activity of the Recombinant AC-like Protein and its Further Functional Characterization

The resultant purified recombinant AC-like protein was then tested for its possible *in vitro* adenylate cyclase activity and in its pure native form using the cAMP-linked enzyme immunoassay kit (Catalog # CA201; Sigma, Missouri, USA). In addition, its *in vitro* enzymatic activity was also further functionally characterized based on its ability to generate cAMP from ATP in the presence of other various ionic and/or chemical elements (ATP, GTP, $Mg^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $CO_3^{2-}$, and $F^-$). The outcomes of this assessment are presented below in Figure 3.8.

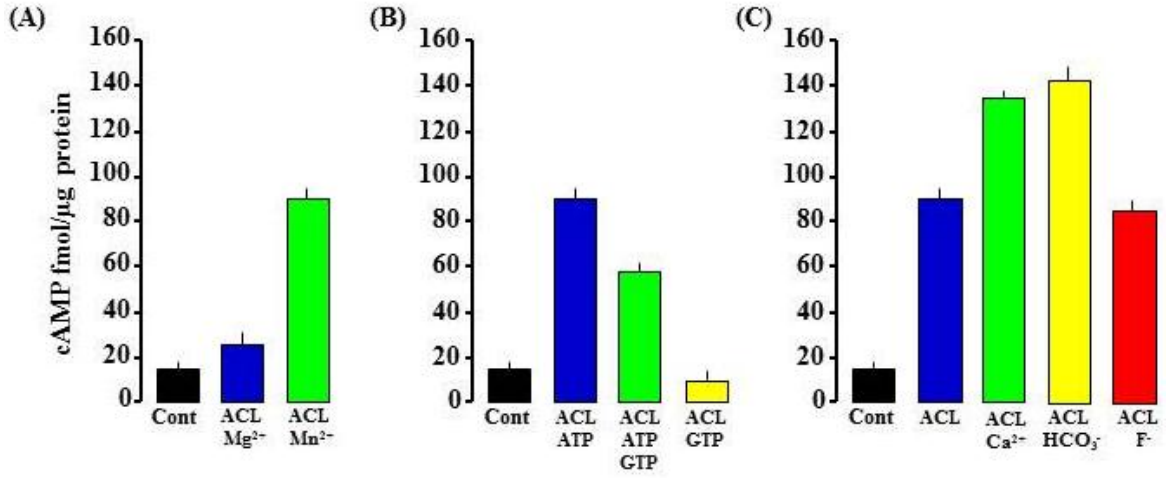


**Figure 3.8: Molecular Characterization of the *In Vitro* Adenylate Cyclase Activity of the Recombinant AC-like Protein.** A reaction mixture containing 10 µg of the purified recombinant AC-like protein, 50 mM Tris-Cl, pH: 8.0, 2 mM IBMX, 5 mM $Mg^{2+}$, 1 mM ATP and/or in the presence of other additives was incubated at room temperature for 20 minutes. The generated cAMP was then measured with a cAMP-specific enzyme immunoassaying kit (Sigma Aldrich Inc., Missouri, USA) based on its acetylation protocol. (A) Cyclic AMP levels generated with the purified recombinant AC-like protein in the presence of magnesium or manganese ions, (B) cAMP levels generated with the purified recombinant AC-like protein in the presence of either ATP and/or GTP, and (C) cAMP levels generated with the purified recombinant AC-like protein in the presence of calcium, bicarbonate or fluoride ions. All assays were undertaken in triplicate sets and the error bars represent the standard errors of the triplicate means (n = 3).

# CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

## 4.1 Discussion

The AC-like protein encoded by the At3g21465 gene in the *Arabidopsis thaliana* is a putative adenylate cyclase (AC) expressed in guard cells and located in the mitochondrion (Gehring, 2010). This gene contains an AC catalytic core motif, but has no annotated domains or yet been shown to contain any known putative AC catalytic function nor does it share any similarities with any annotated and/or experimentally confirmed ACs, but instead, it only appears to be transcriptionally up-regulated in response to biotic stress (Gehring, 2010). In this study, we targeted the AC catalytic region in the AC-like sequence which consists of about 384 bp, where we first retrieved the AC-like gene sequence (1461 bp) from the TAIR site and determined its expressible cDNA sequence.

Specifically, the targeted AC-like gene fragment was isolated from the *Arabidopsis thaliana* ecotype Columbia plants through an amplification of its targeted region with its sequence-specific primers via a specialized RT-PCR system and using the Arabidopsis total mRNA as a template. When resolved on a 1% (w/v) agarose gel, the expected gene fragment of an approximate size of 384 bp was obtained (Figure 3.1). After its successful amplification, the AC-like gene fragment was then ligated into the pTrcHis2-TOPO expression vector to produce a pTrcHis2-TOPO:AC-like gene fragment expression construct. The successful cloning system of this targeted AC-like gene fragment into the pTrcHis2-TOPO expression vector was then verified and confirmed by re-amplifying it with its own forward primer and the vector reverse primer. As is shown in Figure 3.2, the expected fusion gene fragment product of approximately 510 bp was obtained. Additionally, this correct cloning system of the AC-like gene fragment into the pTrcHis2-TOPO expression vector was also further confirmed by nucleotide-specific sequencing at the Inqaba Biotechnological Sciences (Pretoria, RSA) (results not shown).

The successfully designed pTrcHis2-TOPO:AC-like gene fragment expression construct was then used to transform some chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells followed by a partial expression of the desired AC-like recombinant protein through an induction of the transformed cells with 1 mM IPTG at an $OD_{600}$ of 0.5. The expressed recombinant protein was then resolved by SDS-PAGE and as expected, a recombinant fusion C-terminus His-tagged protein product of approximately 18.080 kDa was obtained (Figure 3.3.1), and whose specific core amino acid sequences are presented in Figure 3.3.2.

After successfully expressing the desired and targeted recombinant AC-like protein in the chemically competent *E. cloni* EXPRESS BL21 (DE3) pLysS cells, its ability to generate cAMP from ATP within these prokaryotic systems was also assessed and determined using a cAMP-linked enzyme immunoassaying system. Specifically, the assessment was undertaken in non-induced cells, in induced cells as well as in induced cells supplemented with some commonly known AC modulators; 100 µM forskolin or 100 µM dideoxyadenosine (Ehsan *et al*., 1998, Volotovski *et al*., 1998). As is shown in Figure 3.4, cells induced with 1 mM IPTG had a cAMP generation increase of up to 3.5-fold as compared to the un-induced cells while notably, the treatment of induced cells with 100 µM forskolin had no apparent increase in the levels of the generated cAMP. On the other hand, the treatment of induced cells with 100 µM dideoxy-adenosine notably and significantly reduced the levels of cAMP by a factor of more than 1.75. Conceivably and in a related previous study whereby the PSiP coding region of a pollen-specific putative AC from *Agapanthus umbellatus* (*Liliaceae*) was cloned into bacterial cells, treatment of cells with 1 mM IPTG increased the cAMP levels by a factor of 3.0 while treatment of induced cells with 100 µM of the potent AC inhibitor - dideoxyadenosine to the growing pollen tubes, transiently caused a temporary growth arrest that was accompanied by a reduction of the cAMP concentration by a factor of 1.8 (Moutinho *et al*., 2001). All these previous findings are directly and perfectly consistent with our own findings in this study.

In summary and at this point, it could be positively speculated that the partially expressed *A. thaliana* AC-like recombinant protein is either a *bona fide* AC molecule capable of directly converting ATP to cAMP or else it is simply another functional plant molecule capable of stimulating the functional activities of other resident ACs (*E. coli* ACs in this case) to produce cAMP.

Therefore and in order to determine if the partially expressed recombinant AC-like protein had any direct and/or specific functional AC activity, its possible *in vivo* catalytic activity was then assessed through a functional complementation test. This test was performed using a mutant *E. coli* strain (the SP850 *cya*A) that is systematically deficient in endogenous AC activity and therefore cannot ferment lactose (Moutinho *et al*., 2001). When this mutant strain is grown on MacConkey agar, it normally produces white/yellowish colonies as compared to the magenta red/deep purple colonies produced by its wild-type counterpart (Moutinho *et al*., 2001, Ruzvidzo *et al*., 2013; Swiezawska *et al*., 2014). Therefore and in order to test if the anticipated functional activity of the partially expressed recombinant AC-like protein could rescue this mutant strain, cells of this mutant strain were then transformed with the pTrcHis2-TOPO:AC-like gene fragment expression construct followed an assessment of the colony phenotypes on MacConkey agar medium supplemented with 0.1 mM IPTG (Figure 3.5). As is shown in the Figure 3.5, the transformed *cya*A mutant cells stained magenta deep-purple, signifying their rescuing aspect by the used recombinant expression construct from their mutant state into a wild type state and thus unequivocally confirming the recombinant AC-like recombinant protein as a *bona fide* functional higher plant AC.

Consequently and after confirming the recombinant AC-like protein as a *bona fide* functional higher plant AC, its further functional assessment and characterization as a pure product was then sought and undertaken. Notably, the recombinant protein was partially purified on a charged Ni-NTA affinity matrix (Figure 3.6) followed by its recovery as a pure product (Figure 3.7). The purified form of this recombinant was then assessed and further characterized in an *in vitro* AC activity assaying system. As is shown in Figure 3.8, it is apparent that a relatively high level of AC activity (>4 times than control) of the recombinant AC-like protein was demonstrated with a higher preference for the $Mn^{2+}$ metal ion (>3.5-folds) than the $Mg^{2+}$ metal ion as its cofactor for activity. In addition, the recombinant AC-like protein also demonstrated a sole substrate specificity for the ATP as compared to its structural analogue, GTP even though the presence of GTP in an ATP containing reaction system could have had some binding inhibitory effects (~1.3-fold reduction effect). Furthermore, the recombinant AC-like protein also demonstrated that its inherent AC activity could be positively modulated by the calcium

(~1.625-folds) and bicarbonate (~1.75-folds) ion but with no significant response to the fluoride ion.

Notably, all these observations are neither unexpected nor unusual because in all living organisms, the AC system is represented by its two forms, the soluble AC (sAC) and the trans-membrane AC (tmAC) (Kamenetsky *et al.*, 2006), both of which are also well represented in the plant system (Lomovatskaya *et al.*, 2008). All sACs are functionally activated by the calcium and bicarbonate metal ions (Chen *et al.*, 2000) but not the fluoride ion and forskolin, which on the other hand are the main activators of all tmACs (Newton and Smith, 2004). Furthermore, the activity of all sACs is strictly dependent onto the $Mn^{2+}$ metal ion as a co-factor (Braun and Dods, 1975) compared to tmACs that flexibly depend on both the $Mn^{2+}$ and $Mg^{2+}$ metal ions (Zippin *et al.*, 2004). In addition, while all tmACs are mediated by the second messenger, cAMP via control mechanisms that are typically regulated by the GTP-binding protein, all sACs are mediated by the second messenger, cAMP via control mechanisms that are specifically regulated by the calcium-binding protein, calmodulin (Kamenetsky *et al.*, 2006).

## 4.2 Conclusions

Findings from this study firmly and unequivocally establishes the Arabidopsis AC-like protein (Atg21465) as a functional soluble AC and thereby becoming the fifth ever such candidate to be identified in higher plants after the *Zea mays* pollen signalling protein (Moutinho *et al*., 2001), the *Arabidopsis thaliana* pentatricopeptide repeat protein (Ruzvidzo *et al*., 2013), the *Nicotiana benthamiana* adenylyl cyclase protein (Ito *et al*., 2014), and the *Hippeastrum hybridum* adenylyl cyclase protein (Swiezawska *et al*., 2014). In addition, this protein also becomes the second ever functional AC molecule to be identified in the *Arabidopsis thaliana* plant after the pentatricopeptide repeat protein (Ruzvidzo *et al*., 2013). Most importantly, these findings have somewhat significantly and for the first time ever, established a functional property for the previously unknown protein (AC-like protein) in the *Arabidopsis thaliana* plant. This specific result on its own is indeed a very novel and elegant outcome with a very huge and massive importance and significance to both our current literature and academic scholarship in the whole broader domain of modern sciences.

## 4.3 Recommendations

Findings from this study permit for the following two possible and practically feasible recommendations:

- Firstly, since this molecule (AC-like protein) was bioinformatically identified together with the other thirteen putative *Arabidopsis thaliana* molecules (Gehring, 2010; Ruzvidzo *et al*., 2013) and this study has now just confirmed it as a functional higher plant AC, it is therefore imperative that the other twelve outstanding putative candidates are also tested to see if they are possibly *bona fide* higher plant ACs.
- Secondly, since the AC-like protein has just been firmly confirmed as a functional AC, it is therefore very essential that its exact physiological roles in cell communication and signal transduction systems are further investigated so that its exact mode of action, particularly in processes like plant stress response and adaptatiom mechanisms, is properly ascertained and firmly established.

## REFERENCE

**Ashton AR and Polya GM.** Cyclic adenosine 3′,5′ monophosphate in axenic rye endosperm cell culture. *Plant Physiology* (1978), **61:** 718-722.

**Bhat SV., Bajwa BS., Dornauer H and de Souza NJ.** Plant effect of plant growth regulators on morphogenesis and forskolin production in *Plectranthus barbatus*. *Tetrahedron Letters* (1977), **19:** 1669-1672.

**Braun T and Dods RF.** Development of a $Mn^{2+}$-sensitive, "soluble" adenylate cyclase in rat testis. *Proceedings of the National Academy of Sciences USA* (1975), **72:**1097-1110.

**Butcher RW., Baird CE and Sutherland EW**. Effects of lipolytic and antilipolytic substances on adenosine 3',5'-monophosphate levels in isolated fat cells. *Journal of Biological Chemistry* (1968), **243:** 1705-1712.

**Chen L., Chetkovich DM., Petralia RS., Sweeney NT., Kawasaki Y., Wenthold RJ., Bredt DS and Nicoll RA.** Stargazing regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* (2000), **408:** 936-943.

**Curvetto N and Delmastro SA.** Biochemical and physiological proposal for stomatal movement. Possible involvement of adenosine3',5'-cyclic monophosphate. *Plant Physiology and Biochemistry* (1990), **28:** 367-378.

**Curvetto N., Darjania L and Delmastro S.** Effect of two cAMP analogues on stomatal opening in *Vicia faba.* Possible relationship with cytosolic calcium concentration. *Plant Physiology and Biochemistry* (1994), **32:** 365-372.

**DeFeo-Jones D., Tatchell K., Robinson LC., Sigal IS., Vass WC., Lowy DR and Scolnick EM.** Mammalian and yeast *Ras* gene products: biological function in their heterologous systems. *Science* (1985), **228:** 179-184.

**Ehsan H., Reichheld, JP., Roef L., Witters E., Lardon F., Van Bockstaele D., Van Montagu M., Inze´ D and Van Onckelen H.** Effect of indomethacin on cell cycle dependent cyclic AMP fluxes in tobacco BY-2 cells. *Federation of the European Biochemical Societies* (1998), **422:** 165-169.

**Field KG., Olsen GJ., Lane DJ., Giovannoni SJ., Ghiselin MT, Raff EC., Pace NR and Raff RA**. Molecular phylogeny of the animal kingdom. *Science* (1988), **1:** 748-753.

**Geng W., Wang Z., Zhang J., Reed BY., Charles YC., Pak and Orson WM**. Cloning and characterization of the human soluble adenylyl cyclase. *American Journal of Cell Physiology* (2005), **288:** C1305-1316.

**Gehring C**. Adenylate cyclases and cAMP in plant signaling past and present**.** *Cell Communication and Signalling* (2010), **8:** 15-20.

**Ito M., Takahashi H., Sawasaki T., Ohnishi K., Hikichi Y and Kiba A.** Novel type of adenylyl cyclase participates in tabtoxinine-β-lactam-induced cell death and occurrence of wildfire disease in *Nicotiana benthamiana. Plant Signaling and Behavior* (2014), **18:** 115-125.

**Kamenetsky M., Middelhaufe S., Bank EM., Levin LR., Buck J and Steegborn C.** Molecular Details of cAMP Generation in Mammalian Cells: A Tale of Two Systems. *Journal of Molecular Biology* (2006), **362(4):** 623-630.

**Kronstad J., De Maria AD., Funnell D., Laidlaw RD., Lee N., de Sa MM and Ramesh M.** Signaling via cAMP in fungi: interconnections with mitogen-activated protein kinase pathways. *Archeology of Microbiology* (1998), **170:** 395-404.

**Lindner E., Dohadwalla AN and Bhattacharya BK.** Comparative study of myocardial inotropic effects and *in vivo* hemodynamic effects of forskolin and isoproterenol myocardial inotropic effect in young lambs. *Arzneimittel-Forschung* (1978), **28:** 284-289.

**Lomovatskaya LA., Romanenko AS., Filinova NV and Salyaev RK.** Detection of soluble adenylyl cyclase isoforms in plants. *Biochemistry and Biophysics* (2008), **420:** 124-126.

**Lowrie DB.** *Mycobacterium microti* may protect itself from intracellular destruction by releasing cyclic AMP into phagosomes. *Nature* (1975), **254:** 600-602.

**McCalla AF**. The challenge of food security in the 21st century. *TAA Newsletter* (1999), **19:** 12-19.

**Metzger H and Lindner E.** Forskolin activates adenylate cyclase activity and inhibits mitosis in *in vitro* in pig epidermis. *ZRCS Medical Sciences* (1981), **9:** 99-107.

**Moutinho A., Hussey PJ., Trewavas AJ and Malho R.** cAMP acts as a second messenger in pollen tube growth and reorientation. *Proceedings of National Academy of Science USA* (2001), **98:** 10481-10486.

**Newton RP and Smith CJ**. Cyclic nucleotides. *Phytochemistry* (2004), **65:** 2423-37.

**Padh H and Venkitasubramanian TA.** Cyclic adenosine 3′,5′-monophosphate in Mycobacteria. *Indian Journal of Biochemistry and Biophysics* (1976), **13:** 413-414.

**Perlman RL and Pastan I.** Cyclic AMP in metabolism. *Biochemical and Biophysical Research Communication* (1969), **37:** 151-160.

**Rall TW., Sutherland EW and Berthet J.** Regulation of cyclic adenosine 3′,5′-monophosphate levels in guinea-pig cerebral cortex by interaction of adrenergic and adenosine receptor activity. *Journal of Biology and Chemistry* (1957), **224:** 463-472.

**Robison GA., Butcher RW and Sutherland EW.** Cyclic AMP. *Annual Review of Biochemistry* (1968), **37:** 149-174.

**Rodbell M.** Regulation of glucagon receptor binding. *Nature Annual Review* (1980), **284:** 17-22.

**Ruzvidzo O., Dikobe BT., Kawadza DT., Mabadahanye GH., Chatukuta P and Kwezi L.** Recombinant expression and functional testing of candidate adenylate cyclase domains. *Method in Molecular Biology* (2013), **1016:** 13-25.

**Seamon K.B., Padgett W and Daly JW.** Forskolin: Unique diterpene activator of adenylate cyclase in membranes and intact cells. *Proceeding of the National Academy of Science USA* (1981), **78:** 3363-3367.

**Smith I.** *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. *Clinical Microbiology Review* (2003), **16:** 463-496.

**Stuhmer W., Conti F., Suzuki H., Wang XD., Noda M., Yahagi N., Kubo H and Numa S.** Structural parts involved in activation and inactivation of the sodium channel. *Nature* (1989), **339:** 597-603.

**Swiezawska B., Jaworski K., Pawelek A., Grzegorzewska W., Szewczuk P and Szmidt-jaworska A.** Molecular cloning and characterization of a novel adenylyl cyclase gene, HpAC1, involved in stress signaling in *Hippeastrum hybridum*. *Plant Physiology and Biochemistry* (2014), **80:** 41-52.

**Tesmer JJ., Carmen W., Dessauer RK., Roger K., Sunahara LD, Roger A., Johnson Alfred G., Stephen R and Gilman T.** Molecular basis for *P*-site inhibition of adenylyl cyclase. *Biochemistry* (2000), **39:** 464-471.

**Tezuka T., Hiratsuka S and Takahashi SY.** Promotion of the growth of self-incompatible pollen tubes in lily by cAMP. *Plant Cell Physiology* (1993), **34:** 955-958.

**The *Arabidopsis* Genome Initiative.** Analysis of the genome sequence of the flowering plant, *Arabidopsis thaliana*. *Nature* (2000), **408:** 796-815.

**Tsuruhar A and Tezuka T.** Relationship between the self-incompatibility and cAMP level in *Lilium longiflorum*. *Plant Cell Physiology* (2001), **42:** 1234-1238.

**Ullmann A and Danchin A**. Advances in cyclic nucleotide research *(Raven, New York)*, (1983) **15:** 1-53.

**Volotovski ID., Sokolovsky SG., Molchan OV and Knight MR.** Second messengers mediate increases in cytosolic calcium in tobacco protoplasts. *Plant Physiology* (1998), **117:** 1023-1030.

**Zippin JH., Farrell J., Huron D., Kamenetsky M., Hess KC., Fischman D A., Levin LR and Buck J.** Bicarbonate-responsive "soluble" adenylyl cyclase defines a nuclear cAMP micro-domain. *Journal of Cell Biology* (2004), **164:** 527-534.